60 Rec'd PCT/PTO 25 SEP 2001

Form PTO-1390 P21480.P01	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER P21480
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/926218

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/02076	31 March 2000	31 March 1999

TITLE OF INVENTION

SUBSTRATE FOR THIOREDOXIN REDUCTASE

APPLICANT(S) FOR DO/EO/US

Arne HOLMGREN, Marjan H. AMIRI and Hiroyuki MASAYASU

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. X This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ___ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. X This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. X The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. X A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. X is attached hereto (required only if not communicated by the International Bureau).
   b. X has been communicated by the International Bureau.
   c. ___ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. X An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
7. X Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ___ are attached hereto (required only if not communicated by the International Bureau).
   b. ___ have been communicated by the International Bureau.
   c. ___ have not been made; however, the time limit for making such amendments has NOT expired.
   d. X have not been made and will not be made.
8. ___ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))
9. X An oath or declaration of the inventor(s) (35 U.S.C. .371(c)(4)).
   "Unexecuted"
10. ___ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (U.S.C. 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. Assignee: DAIICHI PHARMACEUTICAL CO., LTD., of Tokyo, JAPAN
12. ___ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
13. ___ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
14. X A FIRST preliminary amendment.
    ___ A SECOND or SUBSEQUENT preliminary amendment.
15. ___ A substitute specification.
16. ___ A change of power of attorney and/or address letter.
17. ___ Figure of Drawing to be published_____
18. X Other items or information:

    International Application as publihed (in Japanese).
    PCT/RO/101-PCT EASY (with International Application as filed in Japanese).
    Cover Letter under 35 U.S.C. 371 AND 37 C.F.R. 1.495.
    PCT/IPEA/409 International Preliminary Examination Report (in Japanese).
    PCT/IPEA/408 Written Opinion (in Japanese).
    PCT/IB/308.
    PCT/IB/332.
    PCT/IB/301.
    PCT/IB/304.
    PCT/ISA/210 (in Japanese & English).
    Claim of Priority.

JC03 Rec'd PCT/PTO 25 SEP 2001

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/926218	INTERNATIONAL APPLICATION NO. PCT/JP00/02076	ATTORNEY'S DOCKET NUMBER P21480

19. X The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search report has been prepared by the EPO or JPO. . . . . $ 860.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482). . . . . $ 690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO(37 CFR 1.445(a)(2). . . . . $ 710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO. $1,000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4). . . . . $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ___ 20 ___ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 0.00	

Claims	Number Filed	Number Extra	RATE		
Total Claims	12 - 20 =	0	X $18.00	$ 0.00	
Independent Claims	2 - 3 =	0	X $80.00	$ 0.00	
Multiple dependent claim(s) (if applicable)			+ $270.00	$ 0.00	
			TOTAL OF ABOVE CALCULATIONS =	$860.00	
___ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$ 0.00	
			SUBTOTAL =	860.00	
Processing fee of $130.00 for furnishing the English translation later than ___ 20 ___ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				0.00	
Extension of Time fee in the amount of $				0.00	
			TOTAL NATIONAL FEE =	860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				0.00	
			TOTAL FEES ENCLOSED =	860.00	
			Amount to be refunded	$	
			Charged	$	

a. X A check in the amount of $ 860.00 to cover the above fees is enclosed.

b. ___ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees.

c. X The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0089.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO **CUSTOMER NO. 7055**
AT THE PRESENT ADDRESS OF:

Bruce H. Bernstein
GREENBLUM & BERNSTEIN, P.L.C.
1941 Roland Clarke Place
Reston, VA 20191
(703) 716-1191

Leslie Kanemoto Reg No. 33,329
SIGNATURE

Bruce H. Bernstein
NAME

29,027
REGISTRATION NUMBER

09/926218
JC03 Rec'd PCT/PTO 25 SEP 2001

P21480.A01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Arne HOLMGREN et al.

Serial No : Not Yet Assigned (National Stage of PCT/JP00/02076)

Filed : Concurrently Herewith (International Filing Date March 31, 2000)

For : SUBSTRATE FOR THIOREDOXIN REDUCTASE

PRELIMINARY AMENDMENT

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Prior to calculation of the filing fees and the examination of the above-identified patent application on the merits, the Examiner is respectfully requested to amend the claims as follows:

IN THE CLAIMS

Please amend the claims as follows (a marked-up copy of the claim amendments is provided as an attachment to this Amendment):

3. (Amended-Clean Text) The substrate for thioredoxin reductase according to claim 1 which is reduced by thioredoxin reductase in the presence of NADPH.

Please add new claim 12 as follows:

---12. The substrate for thioredoxin reductase according to claim 2 which is reduced by thioredoxin reductase in the presence of NADPH.---

REMARKS

By the above amendment, claim 3 has been amended and claim 12 has been added to delete multiple dependency.

If there should be any questions, the Examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,
Arne HOLMGREN et al.

Leslie J. Bergman Reg. No. 33,329
Bruce H. Bernstein
Reg. No. 29,027

September 25, 2001
GREENBLUM & BERNSTEIN, P.L.C.
1941 Roland Clarke Place
Reston, VA 20191
(703) 716-1191

MARKED-UP COPY OF AMENDED CLAIMS

3. (Amended) The substrate for thioredoxin reductase according to claim 1 [or claim 2] which is reduced by thioredoxin reductase in the presence of NADPH.

8/pRts 09/926218
JC03 Rec'd PCT/PTO 25 SEP 2001

SPECIFICATION

Substrate for Thioredoxin Reductase

Technical Field

The present invention relates to a substrate for thioredoxin reductase, and an enhancer of peroxidase activity of thioredoxin reductase.

Background Art

The existence of the thioredoxin (hereinafter abbreviated as "TRX" in the specification) /thioredoxin reductase system is known as one of the reduction-oxidation pathway of thiol group. The system regulates reversible reduction-oxidation reaction of thiol group and maintains a constant thiol level in vivo so as to prevent functional depression of thiol protein by formation of disulfide bonds and advancement of peroxidation state.

It has been elucidated that thioredoxin reductase has activity of reductively cleaving a disulfide bond of a target protein in the presence of NADPH and thioredoxin, as well as a variety of other physiological activities. Thioredoxin, a substrate for thioredoxin reductase, is a protein containing having two thiol groups in the molecule, and functions also as a proton donor in reduction of ribonucleotide by ribonucleotide reductase.

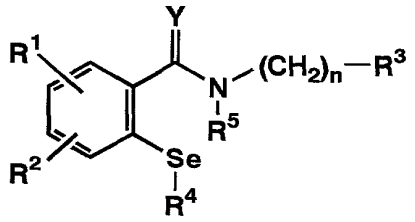
Disclosure of the Invention

An object of the present invention is to provide substances which function as a substrate for thioredoxin reductase and can activate the thioredoxin/thioredoxin reductase system. In particular, the object is to provide a substance which can enhance peroxidase activity proceeded by thioredoxin reductase.

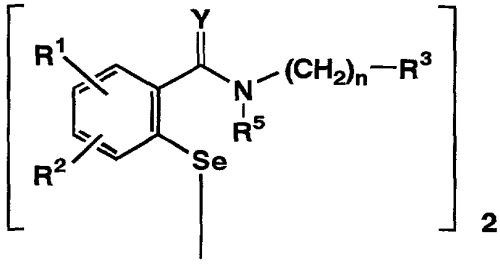
The inventors of the present invention conducted intensive studies to achieve the foregoing object. As a result, they found that selenium compounds such as 2-phenyl-1,2-benzisoselenazol-3(2H)-one can function as substances of thioredoxin reductase by repeated self reduction-oxidation similarly to thioredoxin in the thioredoxin/thioredoxin reductase system, and that the compounds can remarkably enhance peroxidase activity of thioredoxin reductase in the presence of thioredoxin

reductase and thioredoxin. The present invention was achieved on the basis of these findings. It is known that the aforementioned substances can reduce a peroxide (active oxygen) by glutathione peroxidase-like activity (Muller, A. et al., Biochem. Pharmacol., 33, pp.3235-3239). However, the reduction of a peroxide by glutathione peroxidase is based on totally different mechanism from that proceeded by thioredoxin reductase.

The present invention thus provides a substrate for thioredoxin reductase which comprise a substance selected from the group consisting of a compound represented by the following general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



(1)



(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a-S-glutathione group, a -S-α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6

alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

According to preferred embodiments of the aforementioned invention, there are provided the substrate for thioredoxin reductase which comprises a substance selected from the group consisting of 2-phenyl-1,2-benzisoselenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof; and the substrate for thioredoxin reductase which are reduced by thioredoxin reductase in the presence of NADPH.

According to another aspect, there is provided an enhancer of peroxidase activity of thioredoxin reductase which comprise a substance selected from the group consisting of a compound represented by the aforementioned general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof. As a preferred embodiment of the aforementioned invention, there is provided the enhancer which comprises a substance selected from the group consisting of 2-phenyl-1,2-benzisoselenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

According to further aspects of the present invention, there are provided a catalyst comprising a substance selected from the group consisting of a compound represented by the aforementioned general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof which oxidizes reduced thioredoxin in the peroxidase reaction of thioredoxin reductase; a reducing agent comprising the aforementioned substance which reduces a peroxide by oxidizing reduced thioredoxin in the peroxidase reaction of thioredoxin reductase; and an antioxidant comprising the aforementioned substance which prevents peroxidation of a substance in vivo by oxidizing reduced thioredoxin in the peroxidase reaction of thioredoxin reductase.

There are also provided a use of a substance selected from the group consisting of a compound represented by the aforementioned general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof as the aforementioned substrate, as the aforementioned enhancer of

peroxidase activity of thioredoxin reductase, as the aforementioned catalyst, as the aforementioned reducing agent, and as the aforementioned antioxidant; a use of a substance selected from the group consisting of a compound represented by the aforementioned general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof for the manufacture of the aforementioned substrate, the aforementioned enhancer of peroxidase activity of thioredoxin reductase, the aforementioned catalyst, the aforementioned reducing agent, or the aforementioned antioxidant.

In addition to these inventions, there are provided a method for enhancing peroxidase activity of thioredoxin reductase in vivo which comprises the step of administering an effective amount of a substance selected from the group consisting of a compound represented by the aforementioned general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof to a mammal including a human; a method for reducing a peroxide in vivo which comprises the step of administering an effective amount of the aforementioned substance to a mammal including a human; and a method for preventing peroxidation of a substance in vivo which comprises the step of administering an effective amount of the aforementioned substance to a mammal including a human.

Brief Explanation of the Drawings

Fig. 1 shows reduction of Compound A (2-phenyl-1,2-benziso-selenazol-3(2H)-one, ebselen) by human thioredoxin reductase.

Fig. 2 depicts reduction of compound A by thioredoxin reductase. (A) shows reduction of compound A with a low concentration of thioredoxin reductase, and (B) shows generation of selenol groups detected by DTNB after reduction of Compound A by thioredoxin reductase for 10 min. In the figures, Ebselen means compound A.

Fig. 3 shows effect of human thioredoxin on reduction of compound A by thioredoxin reductase.

Fig. 4 depicts oxidation of E.coli Trx-$(SH)_2$ by compound A determined by fluorescence spectroscopy (Fig.4 (A)), and the decreasing ratio of the fluorescence emission at 340 nm following the mixing of 0.1 μM Trx-$(SH)_2$ and 0.1 μM compound A (Fig.4 (B)),. In the figures, Trx represents thioredoxin and Ebse compound A.

Fig. 5 shows reduction of hydrogen peroxide by human thioredoxin reductase and effect of compound A and thioredoxin. In the figure, Trx, EbSe and TrxR represent thioredoxin, compound A and thioredoxin reductase, respectively.

Fig. 6 shows effect of thioredoxin and compound A on reduction of hydrogen peroxide by thioredoxin reductase. In the figure, Trx represents thioredoxin and EbSe compound A.

Fig. 7 shows effect of hydrogen peroxide concentrations on activity of thioredoxin with compound A. In the figure, Trx, TrxR and EbSe represent thioredoxin, thioredoxin reductase and compound A, respectively.

Fig. 8 shows effects of compound A on reduction of hydrogen peroxide. In the figure, Ebselen means compound A.

Best Mode of Carrying Out the Invention

As the C_1-C_6 alkyl group represented by R^1 and R^2, either a straight or a branched chain alkyl group may be used, and examples include methyl group, ethyl group, n-propyl group, isopropyl group, cyclopropyl group, n-butyl group, sec-butyl group, isobutyl group, tert-butyl group, n-pentyl group, and n-hexyl group. As the C_1-C_6 alkoxyl group represented by R^1 and R^2, either a straight or a branched chain alkoxyl group may be used, and examples include methoxy group, ethoxy group, n-propoxy group, isopropoxy group, n-butoxy group, sec-butoxy group, tert-butoxy group, n-pentoxy group, and n-hexoxy group.

As the aryl group represented by R^3, for example, a monocyclic to a tricyclic, preferably a monocyclic or a bicyclic aryl group having 6 to 14 carbon atoms, preferably 6 to 10 carbon atoms can be used. More specifically, phenyl group or naphthyl group and the like are preferred. As the aromatic heterocyclic group represented by R^3, for example, a monocyclic to a tricyclic, preferably a monocyclic or a bicyclic aromatic heterocyclic group containing one ore more heteroatoms such as nitrogen atom, oxygen atom and sulfur atom can be used. When two or more heteroatoms are contained, they may be same or different. Examples include thienyl group, furyl group, pyrrolyl group, imidazolyl group, pyrazolyl group, isoxazolyl group, pyridyl group, pyrazinyl group, pyrimidinyl group, pyridazinyl group, indolizinyl group, isoindolyl group, indolyl group, isoquinolyl group, quinolyl group, phthalazinyl

group, naphthylidinyl group, quinoxalinyl group, quinazolinyl group, cinnolinyl group, pteridinyl group, carbazolyl group, acridinyl group, phenanthridinyl group, and phenothiazinyl group.

The aryl group, the aromatic heterocyclic group, the 5- to 7-membered cycloalkyl group, or the 5- to 7-membered cycloalkenyl group represented by R^3 may have one or more substituents on the ring. When the ring is substituted with two or more substituents, they may be same or different. The position of the substituent is not particularly limited, and the substituent may be present at any position on the ring. The type of the substituent is not particularly limited, and examples include a C_1-C_6 alkyl group, a C_2-C_6 alkenyl group, a C_2-C_6 alkynyl group, a C_6-C_{14} aryl group, a heterocyclic group (the heterocycle used herein includes aromatic heterocyclic groups and partially saturated or saturated heterocyclic groups), a halogen atom (the halogen atom used herein may be any one of fluorine atom, chlorine atom, bromine atom, or iodine atom), hydroxyl group, oxo group, amino group, ammonium group, imino group, mercapto group, thioxo group, cyano group, nitro group, carboxyl group, phosphate group, sulfo group, hydrazino group, a C_1-C_6 ureido group, a C_1-C_6 imido group, isothiocyanate group, isocyanate group, a C_1-C_6 alkoxyl group, a C_1-C_6 alkylthio group, a C_6-C_{14} aryloxy group, a heterocyclic-oxy group, a C_6-C_{14} arylthio group, a heterocyclic-thio group, a C_7-C_{15} aralkyl group, a heterocycle-alkyl group, a C_7-C_{15} aralkyloxy group, a heterocyclic-alkyloxy group, a C_1-C_6 alkoxycarbonyl group, a C_6-C_{14} aryloxycarbonyl group, a heterocyclic-oxycarbonyl group, a C_2-C_7 alkylcarbonyl group, a C_6-C_{14} arylcarbonyl group, a heterocyclic-carbonyl group, a C_2-C_7 alkylcarbonyloxy group, a C_6-C_{14} arylcarbonyloxy group, a heterocyclic-carbonyl oxygroup, a C_2-C_8 alkylcarbonylamino group, a C_1-C_6 sulfonyl group, a C_1-C_6 sulfinyl group, a C_1-C_6 sulfonylamino group, a C_1-C_6 carbamoyl group, and a C_2-C_6 sulfamoyl group.

The substituents exemplified above may be further substituted with one or more other substituents. Examples of such substituents include a hydroxy-C_1-C_6 alkyl group, a halogenated-C_1-C_6 alkyl group, a mono- or di-C_1-C_6 alkylamino group, a halogenated-C_1-C_6 alkylcarbonyl group, a halogenated-C_6-C_{14} aryl group, a hydroxy-C_6-C_{14} aryl group, and a mono- or di-C_1-C_6 alkylcarbamoyl group. However, the substituents explained above are referred to only for exemplification, and the

substituents used are not limited to these examples.

Although the type of the -S-α-amino acid group represented by R^4 is not particularly limited, the group may preferably be an amino acid residue containing thiol group. The -S-α-amino acid residue may be a residue of an amino acid which constitutes a protein or a peptide compound. The type of proteins or peptide compounds is not particularly limited so far as they are physiologically acceptable. For example, serum protein such as albumin and globulin may preferably be used. Among serum protein, albumin is more preferred, and human albumin is particularly preferred. Examples of the aralkyl group represented by R^4 whose aryl moiety may optionally be substituted with one or more substituents include benzyl group, parahydroxybenzyl group, and 2,4-dihydrobenzyl group. R^4 and R^5 may combine together to represent single bond, and in that case, a 5-membered ring is formed which contains the nitrogen atom bound to R^5 and the selenium atom. As the C_1-C_6 alkyl group represented by R^5, those exemplified above can be used.

As the substrate for thioredoxin reductase of the present invention, physiologically acceptable salts of the compounds represented by the aforementioned general formula (1) or (1') may be used. The physiologically acceptable salt can suitably be chosen by the person skilled in the art. Hydrates of the compounds as free form or physiologically acceptable salts may also be used. When the compound represented by the aforementioned general formula (1) or (1') has one or more asymmetric carbon atoms, stereoisomers such as optical isomers and diastereoisomers, any mixture of the stereoisomers, racemates and the like may be used as the substrates of the present invention.

Examples of the substrate of the present invention include 2-phenyl-1,2-benzisoselenazol-3(2H)-one (referred to as "ebselen" in the generic name) and S-(2-phenylcarbamoyl-phenylselenyl)albumin. Physiologically acceptable salts or hydrates of these compounds are also preferred as the substrates of the present invention. A method for the preparation of 2-phenyl-1,2-benzisoselenazol-3(2H)-one is disclosed in Japanese Patent Publication (KOKOKU) No. (Hei) 2-38591/1990, and that of S-(2-phenylcarbamoyl-phenylselenyl)albumin in Japanese Patent Unexamined Publication (KOKAI) No. (Hei) 7-233056/1995. Accordingly, by referring to these preparation methods, the person skilled in the art can easily prepare any compound

falling within the scope of the aforementioned general formula (1) or (1').

The substrate of the present invention represented by the aforementioned general formula (1) or (1') is reduced by thioredoxin reductase and can enhance peroxidase activity of the thioredoxin reductase. The substrate of the present invention can also function as a catalyst which oxidizes reduced thioredoxin in the peroxidase reaction proceeded by the thioredoxin reductase, and also function as a reducing agent which reduces a peroxide by oxidizing reduced thioredoxin in the peroxidase reaction proceeded by the thioredoxin reductase. In addition, the substrate of the present invention can function as an antioxidant which prevents peroxidation of substances in vivo by oxidizing reduced thioredoxin in the peroxidase reaction proceeded by the thioredoxin reductase.

Therefore, administration of the substrate of the present invention as a medicament to a mammal including a human can enhances the peroxidase reaction proceeded by the thioredoxin reductase in vivo. As a result, peroxidation of substances in vivo can be prevented or peroxides in vivo can be reduced, thereby homeostasis of oxidation-reduction state of thiol proteins and thiol compounds in vivo can be maintained. The medicament comprising the substrate of the present invention as an active ingredient is useful for the preventive and/or therapeutic treatment of diseases caused by abnormal regulation of intracellular oxidation-reduction and diseases with abnormal regulation of intracellular oxidation/reduction (Mattson, M.P. et al., Nature, 382, pp.674-675, 1996). Examples of such diseases include, for example, ischemic organ diseases (brain, heart, liver, kidney, digestive organs and the like), nerve degenerative diseases caused by inappropriate apoptosis induction (Alzheimer's disease, Parkinson's disease, Huntington's chorea, familial amyotrophic lateral sclerosis [ALS], AIDS and the like), radiation injury, malignant tumor (leukemia etc.), and various inflammatory diseases and endotoxin shock.

Although it is not intended to be bound by any specific theory, the relation between oxidation stress and ischemic organ diseases, various inflammation or endotoxin shock has been recognized, and the participation of inappropriate apoptosis induction in these ischemic organ diseases has been revealed in recent years (Hockonbery, D.M. et al., Cell, 75, pp.241-251, 1993). In the process of inducing

apoptosis, it is known that generation of intracellular peroxides (active oxygen) due to various factors, particularly hydrogen peroxide, triggers the activation of intracellular nucleoprotein transcription factor NF-κB, that is, release of suppressive protein IκB from NF-κB is started and then the programmed cell death (apoptosis) is induced (Frank, J.T. et al., Proc. Natl. Acad. Sci. USA., 87, pp.9943-9947, 1990).

The NF-κB is also under reduction-oxidation control by thioredoxin (Hayashi, T. et al., Biol. Chem., 268, pp.11380-11388, 1993). Normally, SH group of NF-κB bound to IκB, i.e., in the inactivated state, forms a S-S bond and cannot be approached by thioredoxin due to hindrance of IκB. Accordingly, even if IκB is released by activation of NF-κB through stimulations, the oxidized NF-κB cannot bind to DNA. However, when thioredoxin reduces the S-S bond of the NF-κB to form NF-κB as the activated form, the activated NF-κB migrates into the nucleus and binds to DNA, and then activates genes to induce apoptosis and various inflammatory reactions. Therefore, the substrate of the present invention is expected to participate in suppression of the reduction by Trx.

When the substrate of the present invention is used as a medicament, the substance selected from the group consisting of the compound represented by the aforementioned general formula (I) or (I') and the physiologically acceptable salt thereof, and the hydrate thereof and the solvate thereof, per se, may be administered. Generally, it is preferred to prepare and administer a pharmaceutical composition containing the aforementioned substance as an active ingredient together with one or more pharmaceutical additives. As the pharmaceutical additive, for example, vehicles, binders, disintegrants, and solubilizers can be used, and two or more types of pharmaceutical additives may be used in combination. The form of the pharmaceutical composition is not particularly limited, and examples include the compositions for oral administration such as tablets, capsules, powders, granules and syrups, and those for parenteral administration such as injections, drip infusions, injections, suppositories, transdermal preparations, preparations for mucous membrane, creams, ointments, nasal drops, eye drops, ear drops and patches. These pharmaceutical compositions can be manufactured according to conventional methods in the art.

A dose of the aforementioned medicament can appropriately be chosen

depending on the conditions such as the type of a disease to be treated, the age and body weight of a patient and severity of the disease. For example, in oral administration, the dose may be in the range of from 0.05 to 5,000 mg (as the amount of the active ingredient) per day for an adult. When a medicament containing 2-phenyl-1,2-benzisoselenazol-3(2H)-one as an active ingredient is used, the dose for oral administration may preferably be in the range of from 100 to 2,000 mg (as the amount of the active ingredient), more preferably in the range of from 200 to 1 ,000 mg per day for an adult. However, the aforementioned dose can appropriately be increased or decreased depending on the aforementioned conditions.

Examples

The present invention will be further explained with reference to examples. However, the present invention is not limited to these examples. In the following examples, compound A represents 2-phenyl-1,2-benzisoselenazol-3(2H)-one (sometimes referred to as "Ebselen" in the figures).

Example 1: Formulation example (Tablet)

Compound A	50 mg
Carboxymethylcellulose	25 mg
Starch	5 mg
Crystalline cellulose	40 mg
Magnesium stearate	2 mg
Total	122 mg

Example 2: Experimental example

(A) Materials and methods

(1) Materials and enzymes

NADPH and DTNB were from Sigma. Hydrogen peroxide (30%) and dimethyl sulfoxide (DMSO) were from Merck. Thioredoxin reductase (TrxR) from calf thymus or human placenta were purified to homogeneity (activity: 25 μmol of NADPH oxidized per min per mg of the enzyme) essentially as described for the rat liver enzyme. Thioredoxin (Trx) from E.coli was a homogeneous preparation and

recombinant human thioredoxin and the mutant C62S/C72S were prepared as described by Ren et al. Compound A was dissolved in dimethyl sulfoxide (DMSO) before experiments.

(2) Spectrophotometric measurements

The activity of enzyme in the presence of compound A was determined for a sample in semimicro quarts cuvettes at room temperature by using a Zeiss PMQ3 spectrophotometer equipped with an automatic sample exchanger and a recorder.

(3) Enzyme assays

Measurements of thioredoxin reductase activity were performed in TF buffer (50 mM Tris-Cl, 1 mM EDTA, pH 7.5) with 100 μM NADPH and a given amount of Compound A. Reactions were carried out with addition of 5 or 10 μl of stock solution of thioredoxin reductase in a final volume of 0.55 ml. Cuvettes used as reference contained the same amount of DMSO as in the samples and also thioredoxin reductase. Absorbance of the control cuvette was automatically subtracted by the spectrophotometer. The reactions were followed at 340 nm.

The activity of thioredoxin reductase was determined in the insulin assay. A mixture of 100 mM potassium phosphate (pH 7.0), 2 mM NADPH, and 0.16 mM insulin was added with Compound A and thioredoxin, and then with thioredoxin reductase in a total volume of 0.55 ml for the reaction. The progress of reduction of insulin disulfides was followed at 340 nm. Generated sulfhydryl or selenol groups were measured at 412 nm by addition of 0.50 ml of a mixture of 6 M guanidine-HCl, 0.20 M Tris-Cl (pH 8.0), 1 mM DTNB and calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$. DTNB reducing activity of thioredoxin reductase by using NADPH was measured at 412 nm in 100 mM potassium phosphate (pH 7.0) containing 10 mM EDTA, 0.2 mM NADPH, 5 mM DTNB and 0.1 mg per ml of bovine serum albumin.

(4) Calculation of selenol groups generated from NADPH oxidations

Compound A absorbs at 340 nm with a 4,000 $M^{-1}cm^{-1}$ molar extinction coefficient, and N-phenyl-2-carboxamidobenzene selenol, the reduction product of selenol by a dithiol, has half the absorption (2000 $M^{-1}cm^{-1}$) at 340 nm. The formation of the compound A-selenol was confirmed by measuring absorption spectra in the presence or absence of excess DTT. In calculations of the formation of compound

A-selenol, a molar extinction coefficient of 8,200 $M^{-1}cm^{-1}$ was used since oxidation of NADPH to $NADP^+$ yields 6,200 $M^{-1}cm^{-1}$.

(5) Fluorescence measurement

Protein fluorescence was measured with a thermostated SPEX-Fluoro Max instrument. Trx-$(SH)_2$ was prepared from E.coli Trx-S_2 640 μM which was incubated at room temperature for 20 min with 10 mM DTT. DTT was subsequently removed by gel chromatography (on a NAP-5 column (Pharmacia) using N_2 equilibrated buffer). Trx-$(SH)_2$ was mixed with Compound A dissolved in a total volume of 3 ml of a mixture (pH 7.5) of 0.1 M potassium phosphate and 1 mM EDTA, and fluorescence was immediately measured in the spectrofluorimeter at 22°C. Excitation of fluorescence was at 290 nm and emission spectra from 300 to 500 nm were recorded. Fluorescence at 340 nm was used to follow the oxidation of Trx-$(SH)_2$ to record the rate of the reaction.

(B) Results

(1) Reduction of compound A by human thioredoxin reductase

It was revealed that compound A was used as a substrate for human thioredoxin reductase because the absorbance at 340 nm decreased rapidly when the pure human thioredoxin reductase (40 nM or 4.5 μg/ml) was added to a cuvette containing 50 or 100 μM of Compound A and NADPH (100μl). The result is shown in Fig. 1. Compound A 50 μM (●) or 100 μM (□) was dissolved in 0.55 ml of a solution containing 50 mM Tris-Cl, 1 mM EDTA (pH 7.5) and 100 μM NADPH, and mixed with 40 nM human thioredoxin reductase. Absorbance at 340 nm was measured and corrected against the blank value (with the same amounts of enzyme, but without compound A). The same experiments using 50 μm (●) and 100 μM (△) Compound A mixed with 17 nM enzyme were performed.

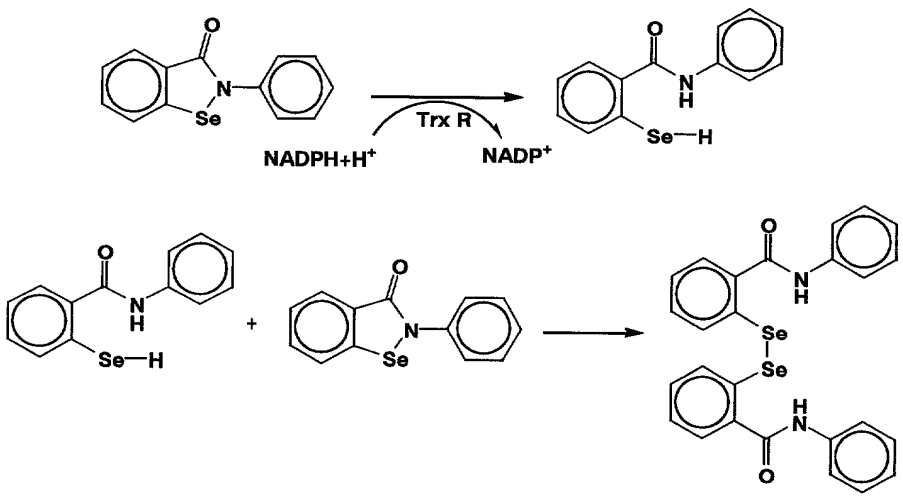
The reaction was fast since with 50 μM Compound A the reaction was complete after 1 min. It was followed by a very slow decrease in the absorbance at 340 nm demonstrating that compound A was not redox cycling with oxygen in contrast to other selenium compounds like selenite or selenocystine. When 6 M guanidine hydrochloride containing DTNB was added to the cuvette at 7 min, an absorbance at 412 nm of 0.400 was measured indicating formation of selenol groups. Compound A

itself gave no reaction with DTNB. The reaction with 100 μM compound A was seemingly slower from the decrease in absorbance at 340 nm using 40 nM enzyme.

A number of experiments with lower concentrations of the enzyme showed a complex change in the absorbance at 340 nm as also seen for 17 nM enzyme and 100 μM compound A (Fig. 1). After an initial decrease, an increase in the absorbance at 340 nm was observed followed by a decrease to give the same value after 15 min as in the sample added with 40 nM enzyme. The result with 7.5 nM enzyme is illustrated in Fig. 2 using 10, 20 50 and 100 μM Compound A. Fig. 2A shows reduction of compound A with a low concentration of thioredoxin reductase. Cuvettes contained 0.55 ml of a solution containing 50 mM Tris-Cl, 1 mM EDTA (pH 7.5), 100 μM NADPH, and 10 μM (●), 20 μM (△), 50 μM (□) or 100 μM (■) Compound A. Each 7.5 nM TrxR was added to the four sample cuvettes. A blank without Compound A at time zero resulted in a decrease in absorbance at 340 nm indicating oxidation of NADPH with 10 μM Compound A. The cuvettes with 50 and 100 μM Compound A showed an increase in absorbance at 340 nm and visible precipitation masked the oxidation of NADPH.

Fig. 2B shows generation of selenol groups detected by DTNB after reduction of Compound A by thioredoxin reductase for 10 min. The same experiment as in Fig. 2A above was repeated for 10 min. Reactions were stopped by addition of 0.5 ml of 6 M guanidine-HCl, 0.20 M Tris-Cl, pH 8.0, 1 mM DTNB, and absorbance at 412 nm was determined and a background of the blank was cancelled to measure selenol groups. The highest concentrations of compound A (50 and 100 μM) gave visible precipitates in the cuvettes. When reactions were stopped by 6 M guanidine hydrochloride and DTNB, all cuvettes contained selenol-like material (Fig. 2B). Apparently, the precipitation masks the decrease in the absorbance at 340 nm resulting from NADPH oxidation and reduction of compound A to the selenol.

Reduction of compound A by NADPH and the enzyme will produce the selenol via an isoselenazolone ring-opened bound intermediate (the following scheme).



Reaction of this intermediate with compound A or reaction of compound A with an enzyme bound intermediate should then produce the diselenide, which has a lower solubility giving rise to the precipitate and increase in absorbance at 340 nm. The diselenide was also reduced to the selenol since addition of 40 nM enzyme to a cuvette with 100 μM compound A and precipitate containing only 4 nM enzyme rapidly cleared the solution and gave the final NADPH oxidation recorded as a variation of the absorbance at 340 nm. The formation of the insoluble diselenide was not a unique feature of the enzyme since it could be mimicked by using a low non-stoichiometric concentration of DTT (10 μM) and 100 μM compound A, whereas excess DTT only gave the selenol as also shown by HPLC.

To determine the K_m- and V_{max} values for compound A, 15 nM enzyme was used with 5, 10 and 20 μM compound A. After 30 seconds, the 5 μM of NADPH was oxidized in all cuvettes, followed by slow increase of the compound A concentrations which may represent diselenide reduction. A K_m-value for compound A below 5 μM was evident and a K_{cal} of 1000$\pm$300 /min was calculated. This makes compound A a substrate of unusual efficiency since human Trx-S_2 has a K_m-value of 2.5 μM and a K_{cal} of 3000 /min.

(b) Effects of Compound A on the enzymatic activity of the mammalian thioredoxin system

To test whether or not Compound A inhibited thioredoxin reductase, enzyme assays were performed. No inhibition was observed with DTNB as substrate using

50 μM Compound A and 10 nM enzyme and only a small effect was seen in an insulin disulfide reduction assay using thioredoxin and thioredoxin reductase (Table 1). The later effect should come from competition with Trx in the assay since compound A did not catalyze insulin disulfide reduction together with the enzyme. Preincubation of the enzyme with compound A in the presence or absence of NADPH did not inhibit the enzyme.

Table 1 shows the effect of compound A on the enzymatic activity of mammalian thioredoxin reductase. (A) shows the result of reactions when mixing 100 nM potassium phosphate (pH 7), 2 mM EDTA, 0.2 mM NADPH, 0.16 mM insulin, 5 μM human Trx and indicated amounts of compound A. The reactions were started by addition of 10 nM calf thymus thioredoxin reductase in the total volume of 0.55 ml of the mixture, and the absorbance at 340 nm was followed for 3 min at 20°C. Then 0.5 ml of a mixture containing 6 M guranidine HCl, 0.20 M Tris-HCl (pH 8.0) and 1 mM DTNB was added to stop the reaction and the absorbance at 412 nm was used to calculate the amount of SH-groups generated in insulin. In (B), 10 nM calf thymus thioredoxin reductase was preincubated with or without 50 μM compound A and 100 μM NADPH for 1 h. Then 10 μl of the resulting solution was added to 500 μl of a mixture of 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA and 5 mM DTNB to determine activity at 412 nm. Activity is expressed as the amount of SH-groups generated (μM) after 3 min.

Table 1

	(A) Trx-catalyzed insulin disulfide reduction			(B) Reduction of DTNB	
Compound A (μM)	0	5	10	0	50
SH-group (μM)	79.8	70.6	68.4	7.3	7.5
Activity (%)	100	89	88	100	103

(3) Effect of thioredoxin on Compound A reduction

Addition of human thioredoxin to thioredoxin reductase, NADPH and Compound A increased the reaction rate. Fig. 3 shows the effect of human thioredoxin on reduction of Compound A by thioredoxin reductase. The oxidation of NADPH by 10 nM TrxR was recorded in the presence of no (●) or 5 μM (◆) of

human Trx-S_2 in 0.5 ml of a mixture containing 50 mM Tris-HCl, 1 mM EDTA (pH 7.5) and 100 μM NADPH. During the first 2 min Trx-S_2 was reduced to Trx-$(SH)_2$. At the arrow compound A was added to both cuvettes. The result demonstrates that Trx-$(SH)_2$ is a fast reductant of Compound A according to the following reaction formulae.

Trx-$(SH)_2$ + Compound A $\rightarrow$ Trx-S_2 + Compound A/selenol

Trx-S_2 + NADPH + H^+ $\rightarrow$ TrxR $\rightarrow$ Trx-$(SH)_2$ + $NADP^+$

(4) Reaction of Compound A with E.coli Trx-$(SH)_2$

Mammalian and E.coli Trx have the same active site GPC and reactivity with disulfides. Since E.coli Trx-$(SH)_2$ has a 3-fold higher tryprophan fluorescence than Trx-S_2, this substance was used to trace the reaction with Compound A. The spectral changes in 0.1 μM Trx-$(SH)_2$ by mixing with 0.1 μM compound A showed oxidation to Trx-S_2. Fig. 4A shows oxidation of E.coli Trx-$(SH)_2$ by compound A determined by fluorescence spectroscopy. N_2 equilibrated 0.1 M potassium phosphate was added with 0.1 μM (1.2 μg/ml) of E.coli Trx-$(SH)_2$ and then with 1 mM EDTA (pH 7.5) to prepare a sample. Fluorescence of the sample was excited at 290 nm. The absorbance at the wavelength range from 300 to 500 nm was recorded. Then 0.1 μM of compound A was added and a spectrum was recorded. Fig. 4B shows the decreasing ratio of the fluorescence emission at 340 nm following the mixing of 0.1 μM Trx-$(SH)_2$ and 0.1 μM compound A. The relative fluorescence emission for 0.1 μM Trx-$(SH)_2$ changed in the deadtime of mixing with 0.1 μM of compound A, indicating an oxidation rate faster than 2×10^7 $M^{-1}s^{-1}$ of Trx-$(SH)_2$. This is the fastest reaction for oxidation of reduced thioedoxin by a low molecular weight compound.

(5) Stimulation of the hydrogen peroxide reductase activity of thioredoxin reductase by compound A

Mammalian thioredoxin reductase directly reduced hydrogen peroxide. Fig. 5 shows reduction of hydrogen peroxide by human thioredoxin reductase and effect of Compound A and thioredoxin. To cuvettes containing 50 mM Tris-HCl, 1 mM EDTA (pH 7.5) and 100 μM NADPH was added 0.5 mM hydrogen peroxide and 17 nM human TrxR (●), 17 nM human TrxR plus 2 μM compound A (△) or 17 nM human TrxR plus 2 μM Compound A and 4.5 μM human Trx (□). The absorbance at 340

nm was determined against a blank with 17 nM thioredoxin reductase but without hydrogen peroxide. As a result, with 0.50 mM hydroperoxide a turnover number of $30 \times min^{-1}$ was calculated. Addition of 2 μM compound A stimulated the activity with the enzyme which increased its activity to a turnover of $450 \times min^{-1}$ or 15-fold. Additional 4.5 μm human Trx increased the activity to a turnover of $900 \times min^{-1}$ or 30-fold. Thus, Compound A acts to dramatically increase the hydrogen peroxide reductase activity of thioredoxin reductase and also acts as a thioredoxin peroxidase mimic.

(6) Effects of Compound A and thioredoxin at high concentration hydrogen peroxide

Addition of 4.5 μM thioredoxin to 17 nM thioredoxin reductase stimulated the reduction of hydrogen peroxide. Fig. 6 shows effect of thioredoxin and Compound A on reduction of hydrogen peroxide by thioredoxin reductase. The same conditions as in Fig. 5 were applied with only 17 nM thioredoxin reductase (●) and addition of 4.5 μM Trx (△). Then 0.5 μM compound A was added to adjust the final compound A concentration to 5.5 μM. Compound A at a low concentration (0.5 μM) increased the reaction rate and 5.5 μM compound A stimulated strongly. Using hydrogen peroxide (2 mM), TrxR (17 nM) and human Trx (5 μM), the same rate or 23 μM min^{-1} of the NADPH oxidation rate was obtained by 1, 2 and 5 μM compound A. Thus, under these conditions the enzyme turnover was 1328 min^{-1} and that of 1 nM Compound A 23 $\times min^{-1}$ demonstrating a highly efficient peroxidase system.

(7) Effects at low hydrogen peroxide concentration

With 2 μM Compound A only 17 nM thioredoxin reductase showed a high activity with 100 μM hydrogen peroxide. Fig. 7 shows effect of hydrogen peroxide concentrations on activity of TrxR with compound A. Determination was performed using 17 nM human thioredoxin reductase and 2 μM compound A (●) or with 17 nM human thioredoxin reductase plus 4.5 μM Trx and 2 μM compound A (△) with the indicated concentrations of hydrogen peroxide. Thus compound A increased activity of the enzyme with lower more physiologically relevant concentrations, and this increase was about 25-fold. Fig. 8 shows effects of compound A on reduction of 100 μm hydrogen peroxide using only 10 nM thioredoxin reductase (●) or 10 nM thioredoxin reductase + 4.5 μM human Trx (△). Activity is expressed as the variation ratio per min, ΔA_{340} /min. The thioredoxin-dependent reaction still

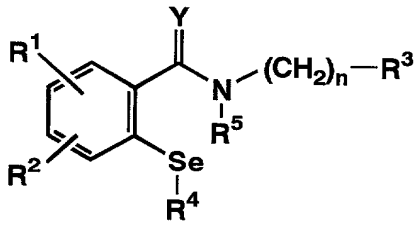
increased, and 100 μM hydrogen peroxide and 1, 2 and 5 μM compound A stimulated the reaction in a similar way both with and without Trx.

Industrial Applicability

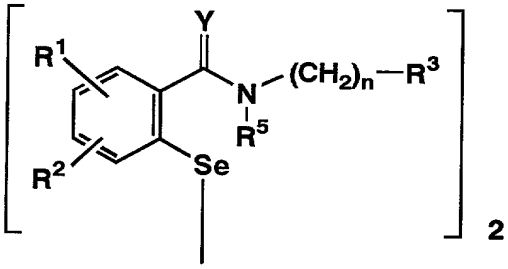
The substrate for thioredoxin reductase of the present invention can activate the thioredoxin/thioredoxin reductase system, in particular, the substrate can enhance peroxidase activity proceeded by thioredoxin reductase. Accordingly, the substrate is very useful as various agents, for example, as an antioxidant which prevents peroxidation of a substance in vivo by oxidizing reduced thioredoxin in the peroxidase reaction of thioredoxin reductase.

What is claimed is:

1. A substrate for thioredoxin reductase which comprises a substance selected from the group consisting of a compound represented by the following general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof:



(1)



(1')

wherein R^1 and R^2 independently represent a hydrogen atom, a halogen atom, a trifluoromethyl group, a nitro group, a C_1-C_6 alkyl group, or a C_1-C_6 alkoxyl group, or R^1 and R^2 may combine together to represent methylenedioxy group; R^3 represents an aryl group, an aromatic heterocyclic group, a 5- to 7-membered cycloalkyl group, or a 5- to 7-membered cycloalkenyl group, and the aryl group, the aromatic heterocyclic group, the cycloalkyl group, and the cycloalkenyl group may be substituted with one or more substituents; R^4 represents a hydrogen atom, a hydroxyl group, a -S-glutathione group, a -S-α-amino acid group, or an aralkyl group whose aryl moiety may be substituted with one or more substituents; R^5 represents a hydrogen atom or a C_1-C_6 alkyl group, or R^4 and R^5 may combine together to represent single bond; Y represents oxygen atom or sulfur atom; n represents an integer of from 0 to 5; and the selenium atom may be oxidized.

2. The substrate for thioredoxin reductase according to claim 1 which comprises a substance selected from the group consisting of 2-phenyl-1,2-benziso-

selenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

3. The substrate for thioredoxin reductase according to claim 1 or claim 2 which is reduced by thioredoxin reductase in the presence of NADPH.

4. An enhancer of the peroxidase activity of thioredoxin reductase which comprises a substance selected from the group consisting of the compound represented by the general formula (I) or (I') and the physiologically acceptable salt thereof, and the hydrate thereof and the solvate thereof according to claim 1.

5. The enhancer according to claim 4 which comprises a substance selected from the group consisting of 2-phenyl-1,2-benzisoselenazol-3(2H)-one or a ring-opened form thereof and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof.

6. A catalyst comprising a substance selected from the group consisting of the compound represented by the general formula (I) or (I') and the physiologically acceptable salt thereof, and the hydrate thereof and the solvate thereof according to claim 1 which oxidizes reduced thioredoxin in the peroxidase reaction proceeded by thioredoxin reductase.

7. A reducing agent comprising a substance selected from the group consisting of the compound represented by the general formula (I) or (I') and the physiologically acceptable salt thereof, and the hydrate thereof and the solvate thereof according to claim 1 which reduces a peroxide by oxidizing reduced thioredoxin in the peroxidase reaction proceeded by thioredoxin reductase.

8. An antioxidant comprising a substance selected from the group consisting of the compound represented by the general formula (I) or (I') and the physiologically acceptable salt thereof, and the hydrate thereof and the solvate thereof according to claim 1 which prevents peroxidation of a substance in vivo by oxidizing reduced thioredoxin in the peroxidase reaction proceeded by thioredoxin reductase.

9. A method for enhancing peroxidase activity of thioredoxin reductase in vivo which comprises the step of administering an effective amount of a substance selected from the group consisting of a compound represented by the general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a

solvate thereof according to claim 1 to a mammal including a human.

10. A method for reducing a peroxide in vivo which comprises the step of administering an effective amount of a substance selected from the group consisting of a compound represented by the general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof according to claim 1 to a mammal including a human.

11. A method for preventing peroxidation of a substance in vivo which comprises the step of administering an effective amount of a substance selected from the group consisting of a compound represented by the general formula (I) or (I') and a physiologically acceptable salt thereof, and a hydrate thereof and a solvate thereof to a mammal including a human.

Fig. 1

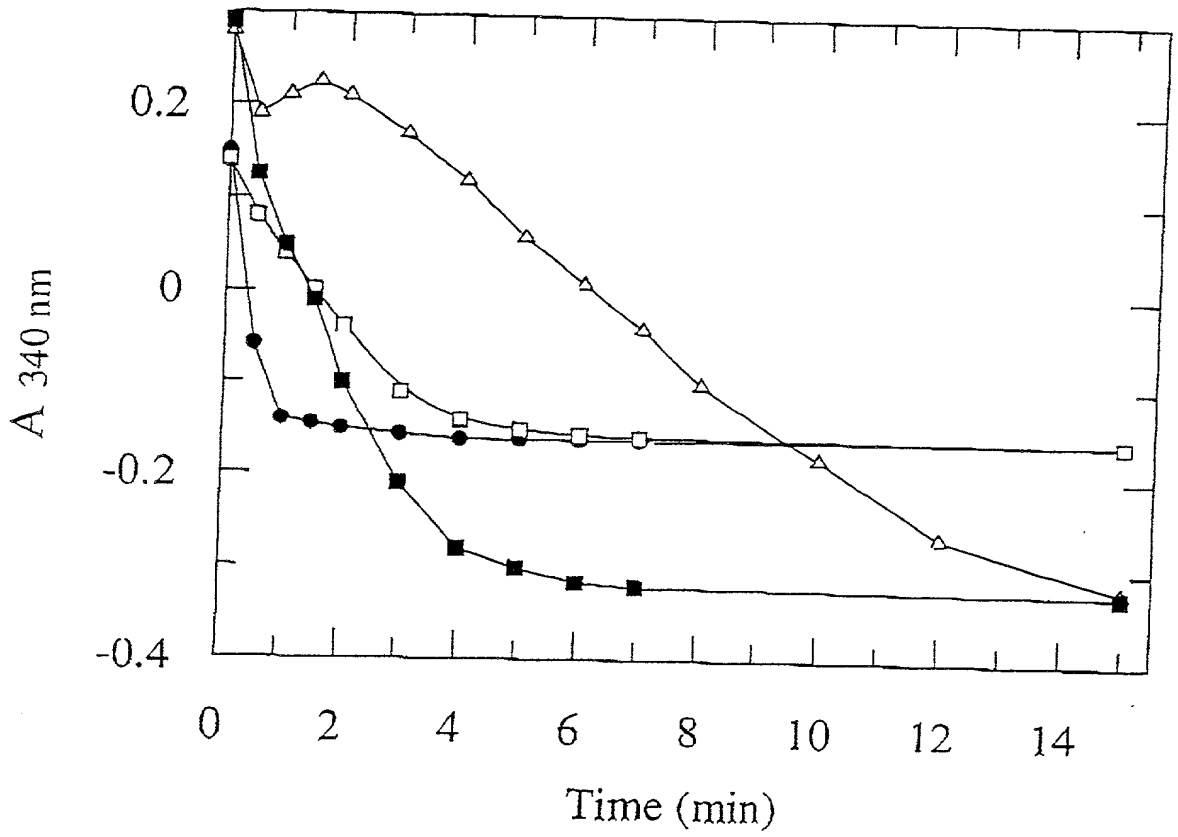
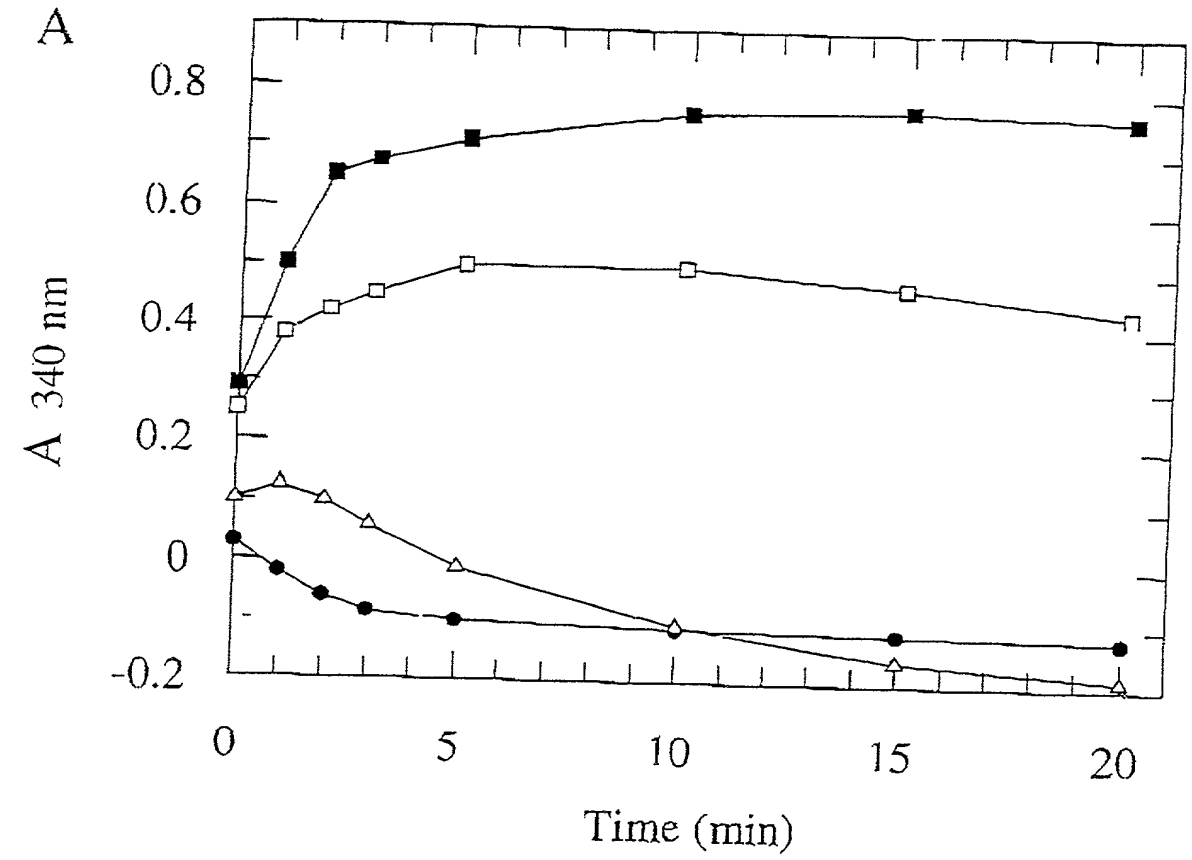


Fig. 2


A
0.8
0.6
0.4
0.2
0
-0.2
A 340 nm
0
5
10
15
20
Time (min)

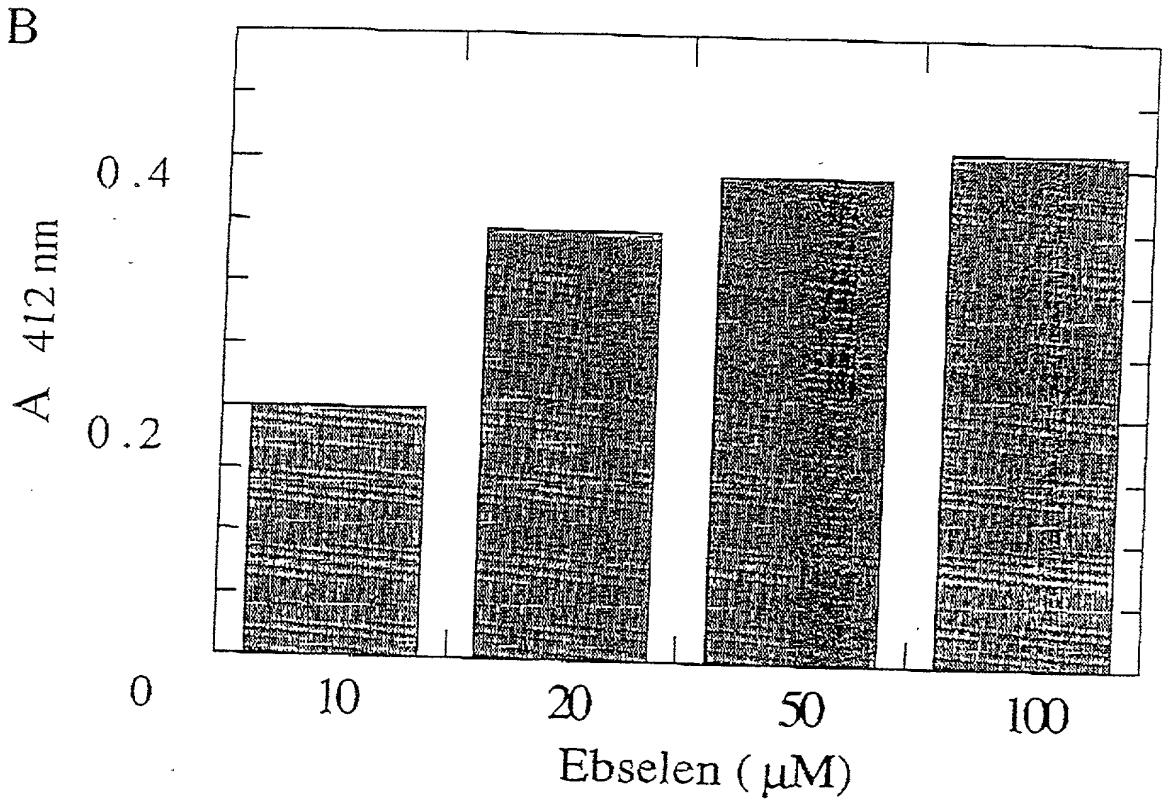

B
0.4
0.2
0
A 412 nm
10
20
50
100
Ebselen (μM)

Fig. 3

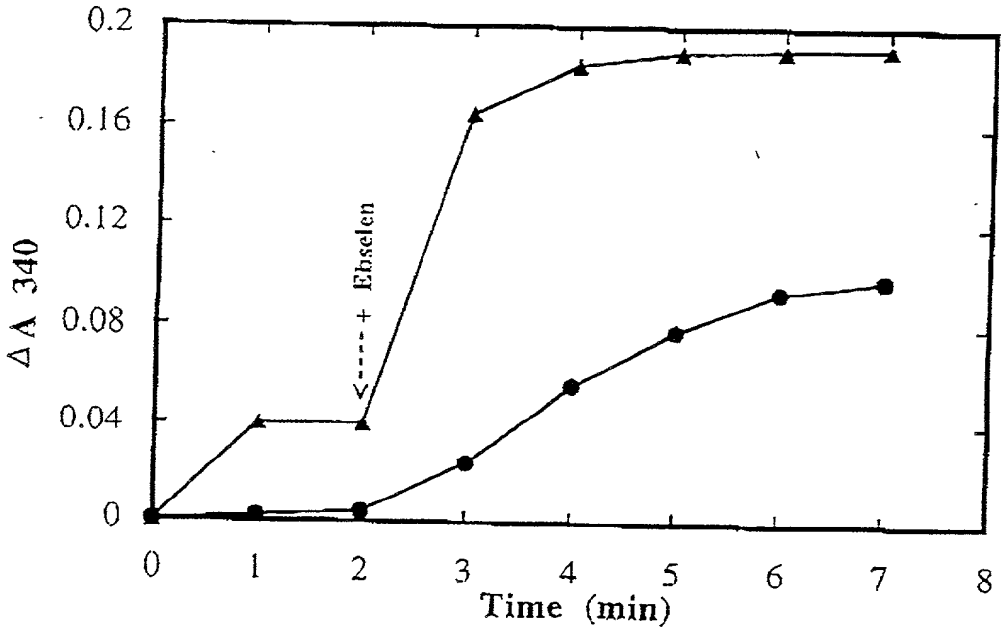
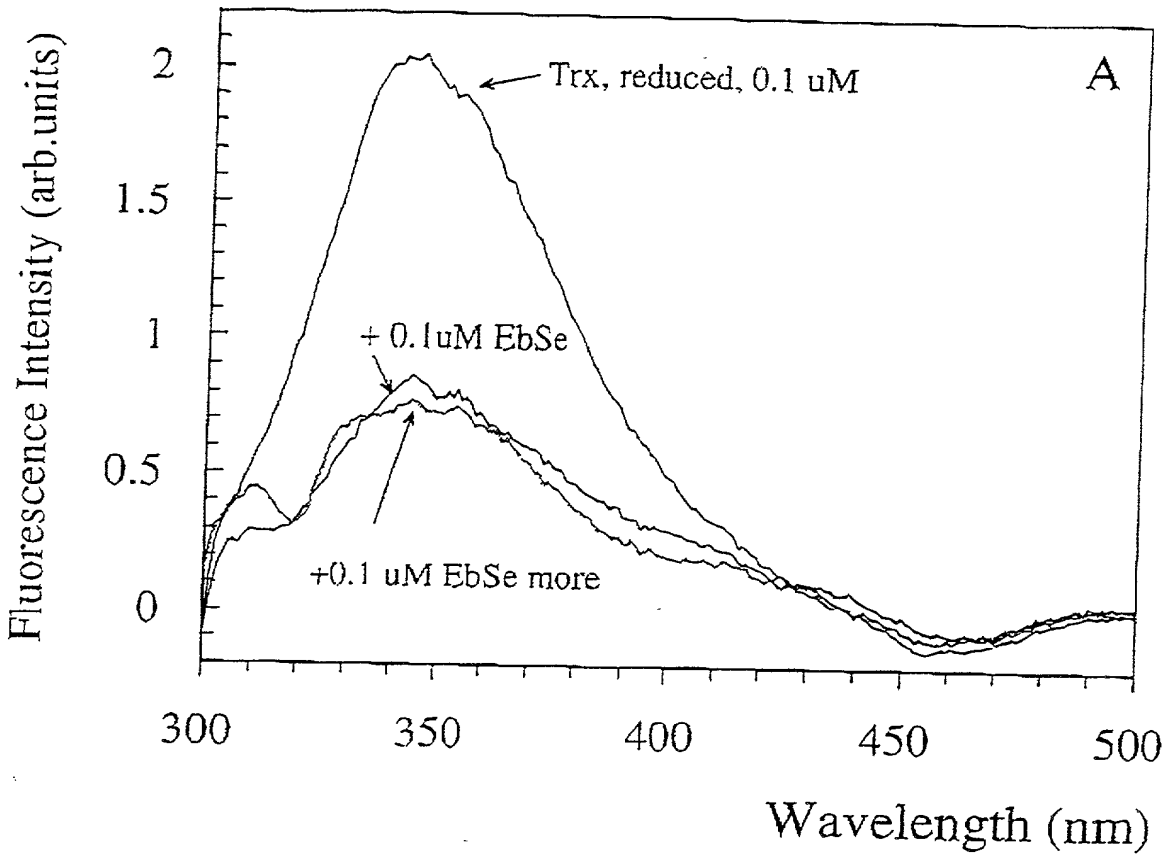


Fig. 4


Trx, reduced, 0.1 uM
A
+ 0.1uM EbSe
+0.1 uM EbSe more
Fluorescence Intensity (arb.units)
2
1.5
1
0.5
0
300
350
400
450
500
Wavelength (nm)

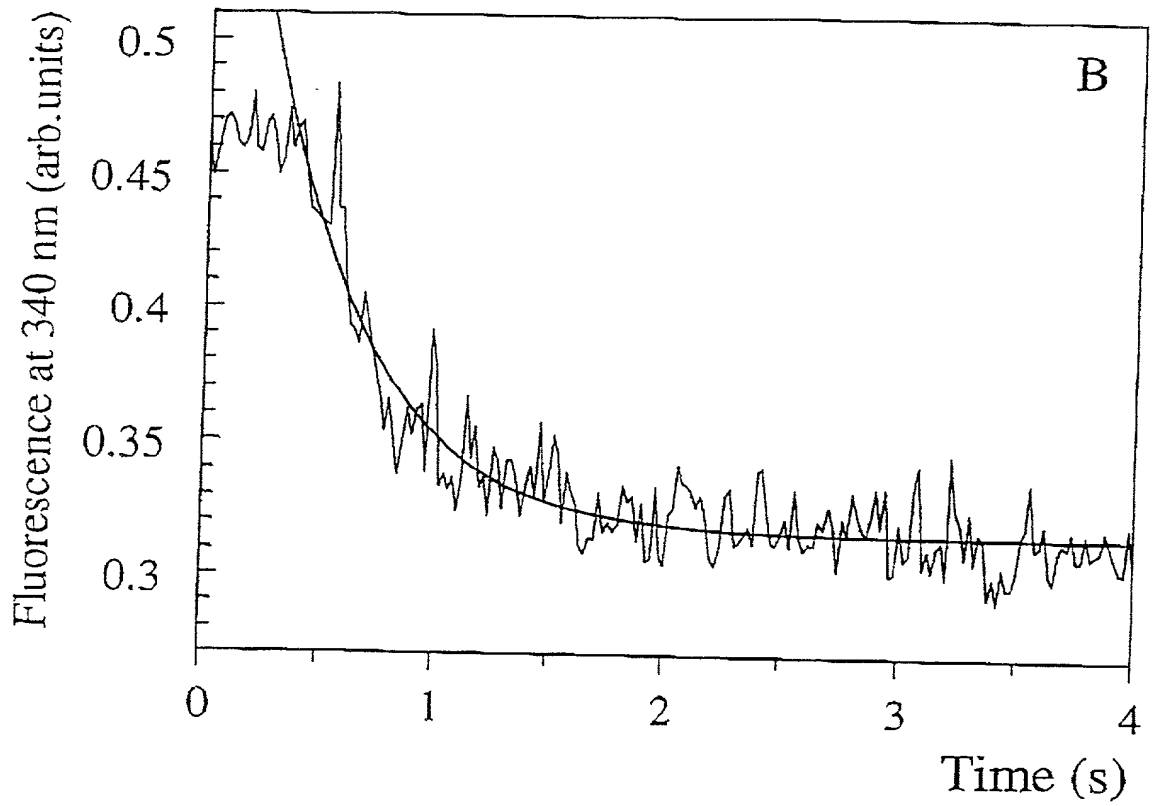

B
Fluorescence at 340 nm (arb.units)
0.5
0.45
0.4
0.35
0.3
0
1
2
3
4
Time (s)

Fig. 5

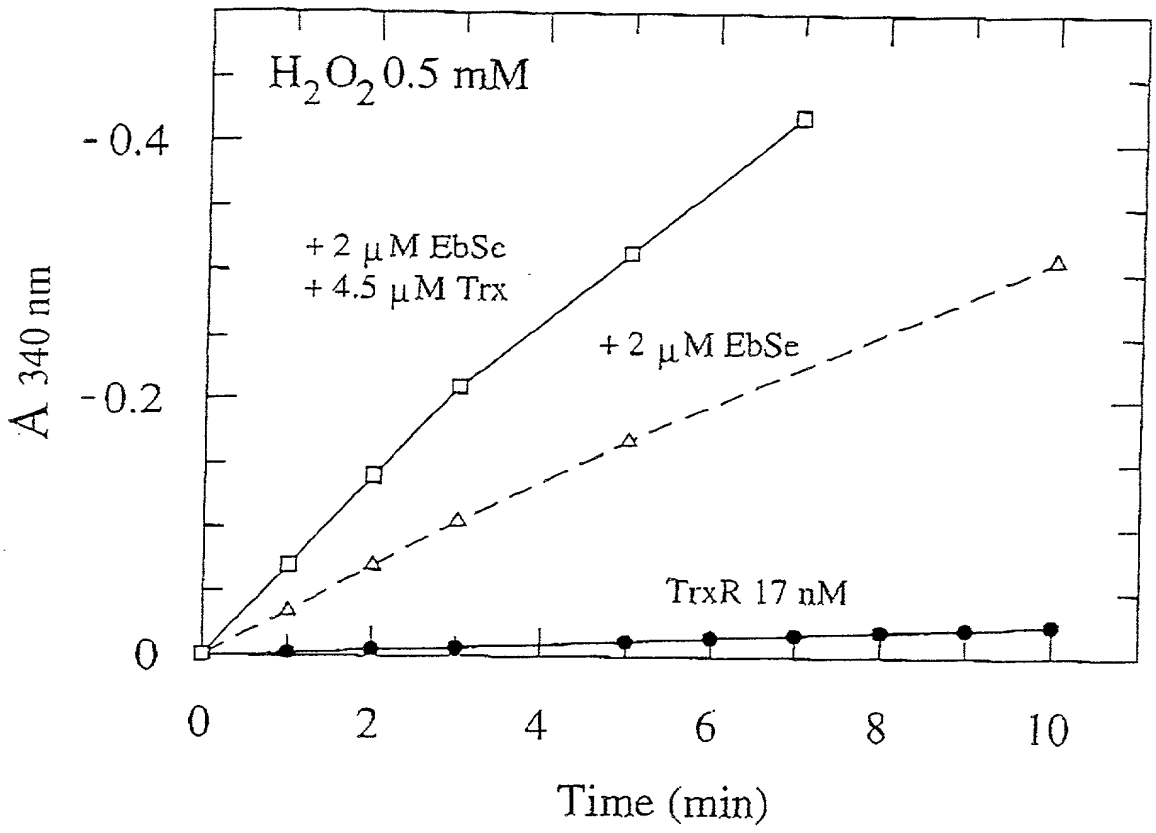

H_2O_2 0.5 mM
+ 2 μM EbSe
+ 4.5 μM Trx
+ 2 μM EbSe
TrxR 17 nM
A 340 nm
-0.4
-0.2
0
0
2
4
6
8
10
Time (min)

Fig. 6

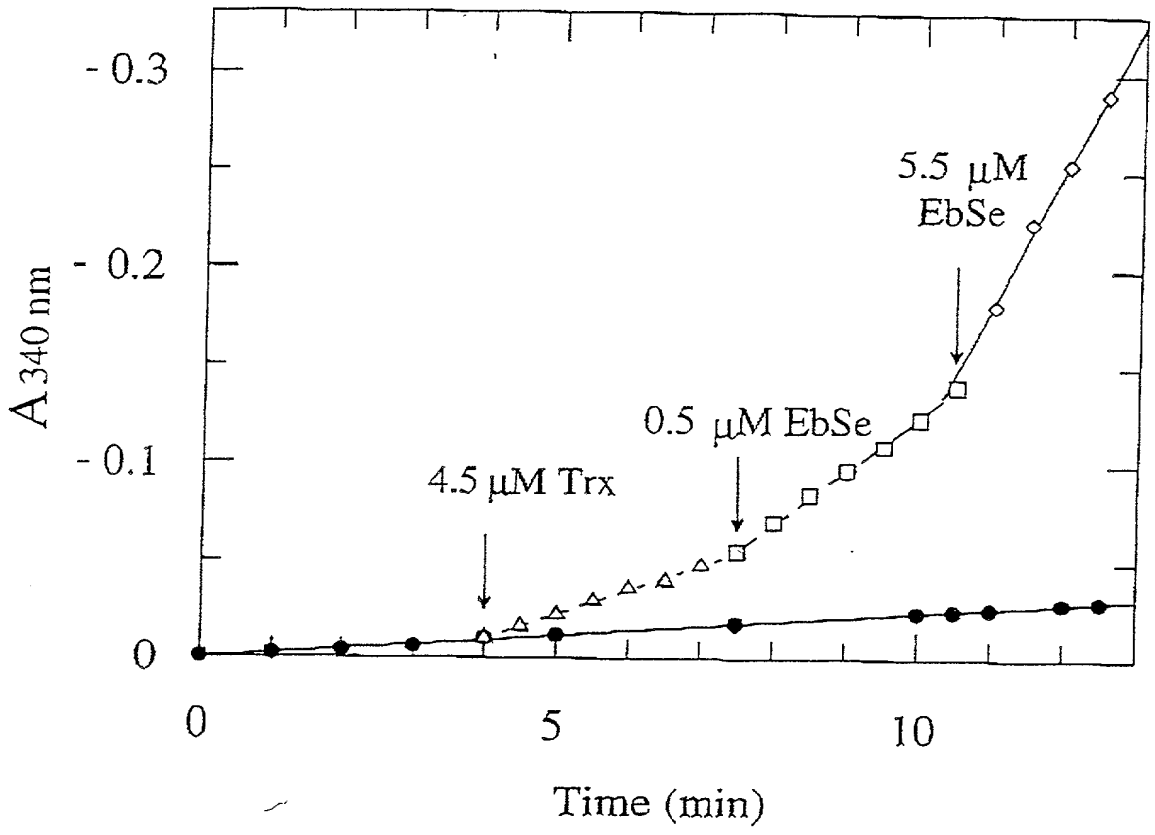


Fig. 7

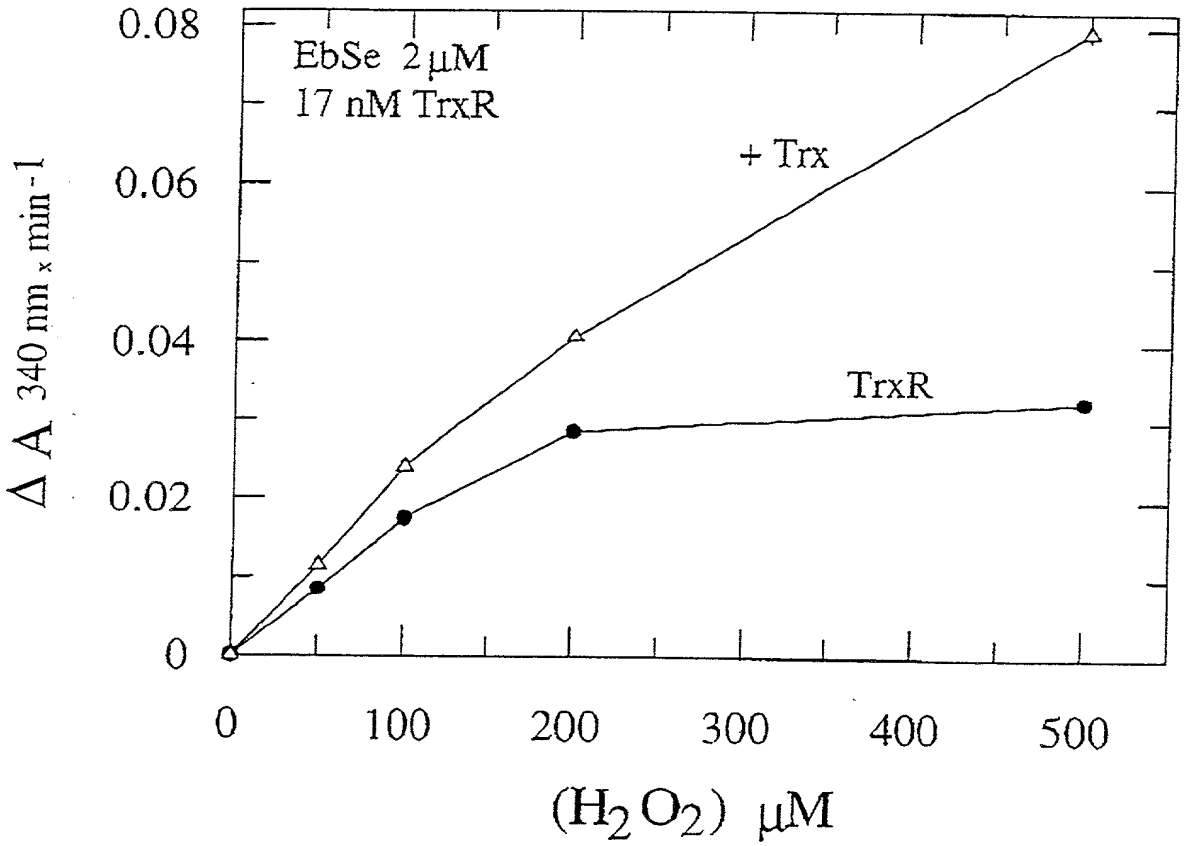
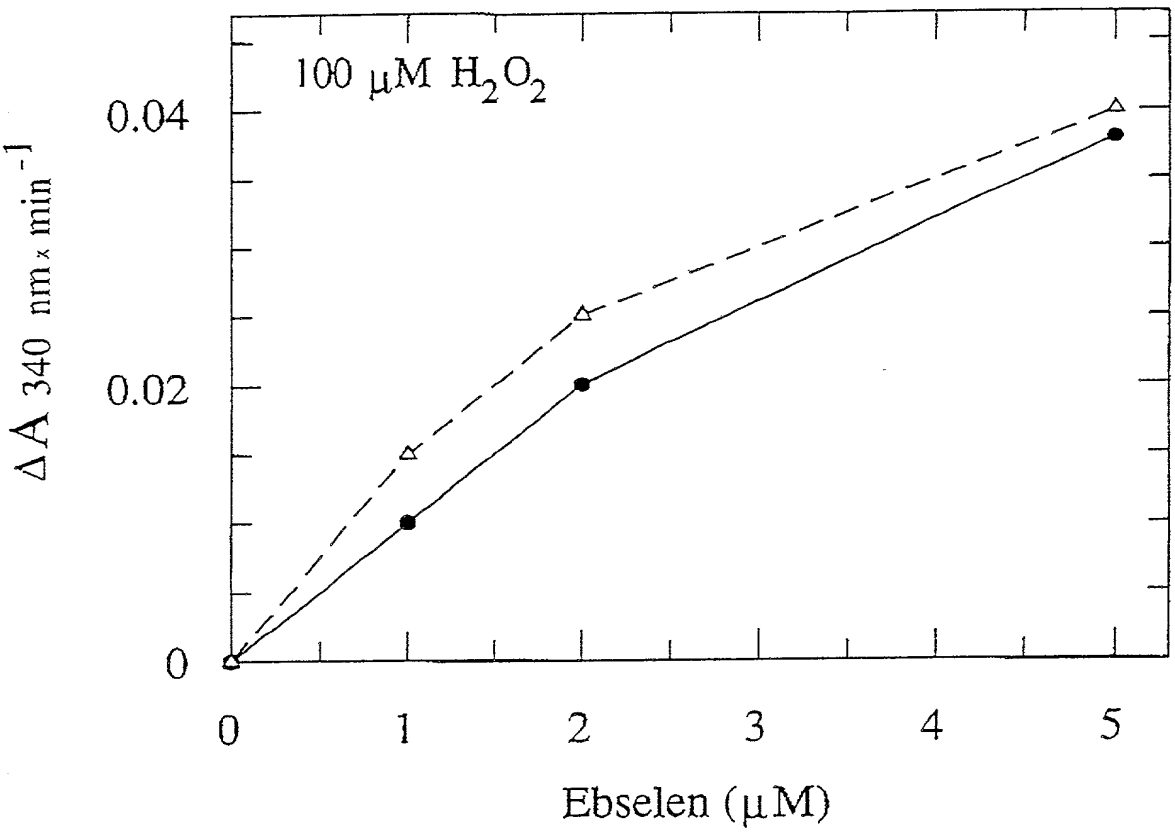

0.08
0.06
0.04
0.02
0
EbSe 2 μM
17 nM TrxR
+ Trx
TrxR
Δ A 340 nm x min-1
0
100
200
300
400
500
(H2O2) μM

Fig. 8



Docket #: p21480.dc1.doc

Declaration and Power of Attorney for Utility or Design Patent Application
特許出願宣言書

Japanese Language Declaration

私は、下欄に氏名を記載した発明者として、以下のとおり宣言する：

私の住所、郵便の宛先および国籍は、下欄に氏名に続いて記載したとおりであり、

名称の発明に関し、請求の範囲に記載した特許を求める主題の本来の、最初にして唯一の発明者である（一人の氏名のみが下欄に記載されている場合）か、もしくは本来の、最初にして共同の発明者である（複数の氏名が下欄に記載されている場合）と信じ、

_____

_____

_____

上記発明の明細書（下記の欄で X 印がついていない場合は、本書に添付）は、

☐ ____年____月____日に提出され、米国出願番号

_____とし、（該当する場合）

____年____月____日に訂正されました。又は、

特許協定条約国際出願番号_____とし、

（該当する場合）____年____月____日に訂正されました。

私は、前記のとおり補正した請求の範囲を含む前記明細書の内容を検討し、理解したことを陳述する。

私は、連邦規則法典第 37 編第 1 条 56 項に定義されているとおり特許資格の有無について重要な情報を開示すべき義務があることを認めます。

私は、合衆国法典第 35 部第 119 条（a-d）項又は第 365 条(b)項に基づく、下記の外国特許出願又は発明者証出願、或いは第 365 条(a)項に基づく、少なくても米国以外の 1 カ国を指名した PCT 国際出願の外国優先権を主張し、更に優先権の主張に係わる基礎出願の出願日前の出願日を有する外国特許出願、又は発明者証出願或るいは PCT 国際出願を以下に“なし”の箱に印をつけることにより明記する：

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Substrate For Thioredoxin Reductase

the specification of which is attached hereto unless the following box is checked:

☒ was filed on **31/Mar/00** as United States Application Number **09/926,218** and was amended on **25/Sep/01** (if applicable) or,

PCT International Application Number **PCT/JP00/02076** and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority under Title 35, United States Code §119(a-d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States, listed below. I have also identified below, by checking the "No" box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

Prior foreign applications 先の外国出願			Priority claimed 優先権の主張
11-92789 (Number) （番号）	**Japan** (Country) （国名）	**31/Mar/99** (Day/Month/Year Filed) （出願の年月日）	☒ Yes ☐ No あり なし
11-101478 (Number) （番号）	**Japan** (Country) （国名）	**08/Apr/99** (Day/Month/Year Filed) （出願の年月日）	☒ Yes ☐ No あり なし

☐ その他の外国特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional foreign application numbers are listed a supplemental priority sheet attached hereto.

Docket #: p21480.dc1.doc

Japanese Language Utility or Design Patent Application Declaration

私は、合衆国法典第 35 部第 119 条(e)項に基づく、下記の合衆国仮特許出願の利益を主張する。

I hereby claim the benefit under Title 35, United States Code §119 (e) of any United States provisional application(s) listed below.

(Application No.)
(出願番号)

(Day/Month/Year Filed)
(出願の年月日)

(Application No.)
(出願番号)

(Day/Month/Year Filed)
(出願の年月日)

(Application No.)
(出願番号)

(Day/Month/Year Filed)
(出願の年月日)

☐ その他の合衆国仮特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

私は、合衆国法典第 35 部第 120 条に基づく下記の合衆国特許出願、又は第 365 条(c)項に基づく合衆国を指名した PCT 国際出願の利益を主張し、本願の請求の範囲各項に記載の主題が合衆国法典第 35 部第 112 条第 1 項規定の態様で、先の合衆国特許出願又は PCT 国際出願に開示されていない限度において、先の出願の出願日と本願の国内出願日又は PCT 国際出願日の間に有効となった連邦規則法典第 37 部第 1 章第 56 条に記載の特許要件に所要の情報を開示すべき義務を有することを認める。

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.)
(出願番号)

(Day/Month/Year Filed)
(出願の年月日)

(現況)
(特許済み、係属中　放棄済み)

(Status)
(patented, pending, abandoned)

(Application No.)
(出願番号)

(Day/Month/Year Filed)
(出願の年月日)

(現況)
(特許済み、係属中　放棄済み)

(Status)
(patented, pending, abandoned)

☐ その他の合衆国又は国際特許出願番号は別紙の追補優先権欄にて記載する。

☐ Additional U.S. or international application numbers are listed on a supplemental priority sheet attached hereto.

私は、ここに自己のに知識に基づいて行った陳述が全て真実であり、自己の有する情報および信ずるところに従って行った陳述が真実であると信じ、さらに故意に虚偽の陳述等を行った場合、合衆国法典第 18 部第 1001 条により、罰金もしくは禁　に処せられるか、またはこれらの刑が併科され、またかかる故意による虚偽による陳述が本願ないし本願に対して付与される特許の有効性を損なうことがあることを認識して、以上の陳述を行ったことを宣言する。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

私、下記署名者は、ここに記載の米国弁護士または代理人に本出願に関し特許商標庁にて取られるいかなる行為に関して、同米国弁護士又は代理人が私に直接連絡なしに私の外国弁護士或るいは法人代表者からの指示を受け取り、それに従うようここに委任する。この指示を出す者が変更の場合には、ここに記載の米国弁護士又は代理人にその旨通知される。

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from either his foreign patent agent or corporate representative, if any, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Docket #: p21480.dc1.doc

Japanese Language Utility or Design Patent Application Declaration

委任状： 私は、下記発明者として、下記に明記された顧客番号を伴う以下の弁護士又は、代理人をここに選任し、本順の手続きを遂行すること並びにこれに関する一切の行為を特許商標庁に対して行うことを委任する。そして全ての通信はこの顧客番号宛に発送される。

顧客番号 7055

現在委任された弁護士は下記の通りである。

Neil F. Greenblum	Reg. No. 28,394
Bruce H. Bernstein	Reg. No. 29,027
James L. Rowland	Reg. No. 32,674
Arnold Turk	Reg. No. 33,094

POWER OF ATTORNEY: As a named inventor, I hereby appoint the attorney(s) and/or agent(s) associated with the Customer Number provided below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

CUSTOMER NUMBER 7055

The appointed attorneys presently include:

Stephen M. Roylance	Reg. No. 31,296
William E. Lyddane	Reg. No. 41,568
William Pieprz	Reg. No. 33,630
Leslie J. Paperner	Reg. No. 33,329

Address: **GREENBLUM & BERNSTEIN, P.L.C.**
1941 Roland Clarke Place
Reston, VA 20191

直接電話連絡先： Direct Telephone Calls to:

GREENBLUM & BERNSTEIN, P.L.C.
(703) 716-1191

唯一のまたは第一の発明者の氏名	Full name of sole or first inventor: Arne HOLMGREN
同発明者の署名 日付	Inventor's signature: [signature] Date: December 21, 2001
住所	Residence: Sollentuna, Sweden SEX
国籍	Citizenship: Sweden
郵便の宛先	Post Office Address: c/o Medical Nobel Institute for Biochemistry, Karolinska Institute
	S-17177 Stockholm, Sweden
第二の共同発明者の氏名（該当する場合）	Full name of second joint inventor, if any: Marjan H. AMIRI
同第二共同発明者の署名 日付	Second Inventor's signature: M. AMIRI Date: December 21, 2001
住所	Residence: Stockholm, Sweden SEX
国籍	Citizenship: Sweden
郵便の宛先	Post Office Address: c/o Medical Nobel Institute for Biochemistry, Karolinska Institute
	S-17177 Stockholm, Sweden

（第三またはそれ以降の共同発明者に対しても同様な情報および署名を提供すること。）

(Supply similar information and signature for third and subsequent joint inventors.)

Docket #: p21480.dc1.doc

Japanese Language Utility or Design Patent Application Declaration

第三の共同発明者の氏名（該当する場合） 3-00	Full name of third joint inventor, if any Hiroyuki MASAYASU
共同発明者の署名 日付	Third Inventor's signature Date Hiroyuki Masayasu January 16, 2002
住所	Residence Tokyo, Japan JPA
国籍	Citizenship Japan
郵便の宛先	Post Office Address c/o Daiichi Pharmaceutical Co., Ltd., Tokyo R & D Center
	16-13, Kita-kasai 1-chome, Edogawa-ku, Tokyo 134-8630, Japan
第四の共同発明者の氏名（該当する場合）	Full name of fourth joint inventor, if any
共同発明者の署名 日付	Fourth Inventor's signature Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address
第五の共同発明者の氏名（該当する場合）	Full name of fifth joint inventor, if any
共同発明者の署名 日付	Fifth Inventor's signature Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address
第六の共同発明者の氏名（該当する場合）	Full name of sixth joint inventor, if any
共同発明者の署名 日付	Sixth Inventor's signature Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address

（それ以降の共同発明者に対しても同様な情報および署名を提供すること。）

(Supply similar information and signature for subsequent joint inventors.)